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Modeling of *Brettanomyces clausenii* Growth on Mixtures of Glucose and Cellobiose

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Abstract

The sequential utilization of glucose and cellobiose during anaerobic fermentations of *Brettanomyces clausenii* was investigated to develop a mathematical model for a simultaneous saccharification and fermentation process. Fermentation of cellobiose was found to be mediated by the presence of extracellular β-glucosidase, which is repressed and inhibited by glucose. A kinetic model containing a number of parameters, which can be determined experimentally, was developed for simulating the dynamics of yeast fermentation of mixtures of glucose and cellobiose. An alternative cybernetic model, which is based on the viewpoint that microbial behavior is the outcome of an instantaneous optimization strategy to maximize their growth rate, is also developed using a much smaller number of parameters. Both models were shown to be similarly capable of simulating the yeast growth dynamics in batch cultures, as well as perturbed cultures with intermittent additions of glucose.

Introduction

Simultaneous cellulose hydrolysis and carbohydrate fermentation to ethanol is an area of active research for the conversion of renewable resources, such as wood from short rotation forests, into liquid fuels such as ethanol. Simultaneous saccharification and fermentation(SSF) is considered advantageous relative to a two step process of saccharification and fermentation since the fermentation of glucose and cellobiose prevents the accumulation of these sugars, thereby reducing the end-product inhibition of the cellulolytic enzymes. Operation, evaluation and improvement of this combined process require a clear understanding of the kinetics of both of the two process steps.

The kinetics of cellulose hydrolysis has been investigated by several researchers. [1] Cellulose hydrolysis by the commonly used cellulase is found to be a two-step process with the exo- and endo-glucanases catalyzing the production of cellobiose and

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β-glucosidase catalyzing the hydrolysis of cellobiose to glucose. The enzymatic reaction rates are variously modeled as modified Michaelis-Menten expressions, with the modifications representing competitive or non-competitive inhibitions by cellobiose and glucose. For any given cellulase system, the type of rate expressions to be used can be determined through initial rate experiments.

The kinetics of yeast fermentation on a mixture of glucose, cellobiose and higher cellodextrins are not as easily determined. To alleviate the end-product inhibition of the glucanases by cellobiose, an organism capable of growing on both glucose and cellobiose, e.g., *Brettanomyces clausenii*, is preferred for the SSF process [2]. Simulation of microbial growth and fermentation dynamics on multiple substrates using unstructured models, such as modified Monod terms or additive Monod terms etc., fails to predict the growth dynamics. This is mainly a result of the complexity of the microbial regulatory processes such as the repression/induction and inhibition/activation of some catabolic enzymes by the catabolites of faster growth supporting substrates.

In this paper, we develop structured models for growth dynamics of the yeast *Brettanomyces clausenii* on mixtures of glucose and cellobiose by incorporating the repression and inhibition of catabolic enzymes into the model equations. Two different approaches are followed for the development of these models: one is the more common kinetic modeling approach which requires the use of a large number of parameters, and the other is a new development in growth modeling known as the cybernetic modeling approach [3,4]. The cybernetic approach assumes the microorganism to be an optimal strategist, i.e., through the regulatory mechanisms of repression/induction and inhibition/activation, the microbial cell maintains some optimal condition which is discussed later. For more background on the cybernetic perspective, the reader is referred to the original literature [3,5]. Both kinetic and cybernetic model simulations are compared with the experimental results.

Materials and Methods

Organism

Parent yeast *Brettanomyces clausenii* (ATCC 10562), which was obtained from NRRL, gave rise to a faster growing, morphologically altered yeast after extended serial SSFs. This faster growing strain was the fermenter in all experiments.

Medium

Yeast Nitrogen Base(YNB) w/o amino acids, a completely defined carbon free medium containing vitamins and a nitrogen source, was obtained from Difco Laboratories and is described elsewhere [6]. Glucose and cellobiose were added to produce concentrated media which were then filter sterilized with disposable Nalgene 0.2μm filters.

Inoculum Preparation

The organism was stored in Nunc cryo tubes at -70° C in a 12% glycerol solution. Inocula were grown in 250-mL shake flasks at 30° C. Inocula media consisted of YNB w/o amino acids with glucose or cellobiose added according to the carbon source used in the fermentor to be inoculated.

Fermentor Description

Experiments were carried out in 5-L Braun Biostat V fermentors with initial culture volumes of 4 L. Temperature was controlled at 30° C and pH was controlled at 5.0. The medium was sparged with nitrogen for 30 minutes immediately following inoculation to remove oxygen.

Sample Analysis

Cell Concentration

Cell dry weight was estimated from absorbance measured at 590 nm. Absorbance varied linearly with cell dry weight up to approximately 0.17. Denser samples were diluted with measured quantitites of sterile media to obtain absorbances below 0.17.

Glucose and Cellobiose Concentrations

Glucose concentrations were determined with a Yellow Springs Instrument Co. glucose analyzer. Cellobiose concentrations were determined by first hydrolyzing the cellobiose through addition of β-glucosidase(Sigma). Samples were incubated for $\geq$ 3 hrs at 30°C. A second glucose assay was done for each sample. The result from the first glucose assay was subtracted from the second and the difference was multiplied by the stoichiometric coefficient to give the cellobiose concentration.

β-glucosidase Activity

β-glucosidase activity was determined via a colorimetric method. Sample aliquots were diluted in a citrate buffer of pH 5.2 and were incubated at 50°C with p-Nitrophenyl β-D-Glucopyranoside(Sigma) for 30 minutes. Incubation was quenched with 2.0 molar sodium carbonate and absorbance at 410 nm was determined.

Results

Figure 1 shows the sugar concentrations and the logarithm of optical density. The growth curve shows an initial lag phase, an exponential growth phase, during which only glucose is consumed, and an immediate second exponential growth phase with a slower growth rate, during which most of the cellobiose is consumed. The slower growth rate during the second growth phase is as expected and in complete agreement with the recently tabulated general characteristics of microbial growth on multiple substrates [7].

A surprising feature of the growth data is in the glucose concentration profile. After the sugar gets completely utilized during the first growth phase, the glucose concentration in the extracellular medium rises sharply during the initial growth on the remaining cellobiose and remains non-zero until cellobiose is almost completely utilized. Also, β-glucosidase assays showed a rapid increase in extracellular activity coincident with the beginning of cellobiose hydrolysis. These results form the basis for an assumption, in our subsequent modeling efforts, that cellobiose utilization by *B. clausenii* is solely through its extracellular hydrolysis to glucose and subsequent utilization of glucose thus formed.

Significant β-glucosidase activity was not found in the cell-free medium during the first growth phase when only glucose was consumed. This observation suggests

a repression of this enzyme synthesis by glucose when glucose is present in sufficient quantities. Near completion of the first growth phase, β-glucosidase synthesis appears to be derepressed due to the lower glucose concentration and induced by cellobiose, resulting in an immediate second growth phase without any intermediate diauxic lag. β-glucosidase activity was not detected after glucose depletion in the absence of cellobiose.

During growth on cellobiose only, i.e., when no glucose is added to the fermentor, the extracellular glucose concentration increases to a significant level during cellobiose hydrolysis: see figure 2. The glucose concentration is significant because it is of the order of the Monod constant for glucose growth and therefore can greatly effect the growth rate. This result further suggests extracellular cellobiose hydrolysis.

Kinetic Modeling

The growth of yeast is assumed to depend on the extracellular glucose concentration in a typical Monod form. As all cellobiose is hydrolyzed extracellularly, the Monod-type dependence on glucose alone will suffice for modeling of growth on cellobiose, provided the enzymatic hydrolysis kinetics are also included in the model equations. The original Monod term is unsatisfactory for simulating the growth dynamics in batch and continuous cultures; such as the initial lag phase or response to perturbations. A simple modification has been used [8] to improve this aspect of batch dynamic simulation by incorporating the intracellular level of a hypothetical key(or rate-determining) enzyme for the catabolism of glucose. Thus,

$$\frac{dC}{dt} = \mu e_1 C \left[\frac{s_1}{K_1 + s_1}\right] \tag{1}$$

where C is the cell concentration (g dw/L), s_1 is glucose concentration (g/L), e_1 is the intracellular key enzyme mass fraction (g enzyme/g cell mass), K_1 is the saturation constant and the product, μe_1, replaces the maximum specific growth rate, $\mu_{1,max}$, which is in the unmodified Monod equation. The rate equation of the intracellular key enzyme fraction can be written as:

$$\frac{de_1}{dt} = \alpha_1 \left[\frac{s_1}{K_{e_1} + s_1}\right] - e_1 \left(\frac{1}{C}\frac{dC}{dt}\right) - e_1\beta_1 \tag{2}$$

where the first term on the right hand side corresponds to its synthesis. Induction occurs through the presence of the sugar s_1. The second term corresponds to a dilution of the intracellular mass due to cell growth [9], and the third term represents first order degradation of the enzyme. From this equation, the maximum value for e_1 during balanced growth phase on s_1, i.e., when $de_1/dt = 0$, in batch cultures can be obtained as:

$$e_{1,max} = \frac{\alpha_1}{\mu_{1,max} + \beta_1} \tag{3}$$

The maximum specific growth rate, $\mu_{1,max}$, which is an experimentally determinable parameter, is the same as $\mu e_{1,max}$ in this structured model. Thus,

$$\mu = \frac{\mu_{1,max}(\mu_{1,max} + \beta_1)}{\alpha_1} \tag{4}$$

If, at the beginning of a batch culture, the intracellular key enzyme fraction is smaller than its maximum balanced growth phase level (possibly due to its degradation during post-exponential or stationary phases of the inoculum culture), then the instantaneous specific growth rate will be proportionally smaller than $\mu_{1,max}$. This situation represents an initial lag phase. The synthesis of e_1 begins immediately provided s_1 is present and the instantaneous specific growth rate reaches its maximum.

The rate equation for s_1 can be written as

$$\frac{ds_1}{dt} = -\frac{\mu e_1 C}{Y}\left[\frac{s_1}{K_1 + s_1}\right] + \nu k_2 e_2 \left[\frac{s_2}{K_2 + s_2}\right]\left[\frac{K_3}{K_3 + s_1}\right] \tag{5}$$

where the first term corresponds to the consumption of s_1 for cell growth, related to the cell growth rate in Eq. 1 by the yield coefficient Y_1. The second term corresponds to the production of ν g glucose from 1g cellobiose. Cellobiose hydrolysis kinetics can be written as:

$$\frac{ds_2}{dt} = -\left[\frac{k_2 e_2 s_2}{K_2 + s_2}\right]\left[\frac{K_3}{K_3 + s_1}\right] \tag{6}$$

where s_2 is the cellobiose concentration, e_2 is the extracellular enzyme concentration, K_2 is the Michaelis-Menten constant, k_2 is the maximum enzymatic reaction rate and K_3 is the dissociation constant for the non-competitive inhibition of this enzyme by glucose. The synthesis rate of β-glucosidase is assumed to be the rate-determining step relative to the secretion rate of this enzyme into the extracellular medium. Hence, the rate equation for e_2 can be represented simply as:

$$\frac{de_2}{dt} = \alpha_2 C\left[\frac{s_2}{K_{e_2} + s_2}\right]\left[\frac{K_4}{K_4 + s_1}\right] - \beta_2 e_2 \tag{7}$$

where the first term in brackets accounts for the induction of enzyme synthesis by cellobiose and the second term in brackets represents the repression of enzyme synthesis by glucose. The last term accounts for any degradation by extracellular proteases or thermal denaturation.

Equations 1,2,5,6 and 7 comprise the kinetic model for the dynamic growth behavior of the yeast *B. Clausenii* on mixtures of glucose and cellobiose. The constants μ_1, K_1 and Y_1 are directly from the standard Monod model for microbial growth and can be estimated readily through initial growth rate experiments. Similarly, the constants k_2 and K_2 relate to the Michaelis-Menten kinetics of cellobiose hydrolysis and are also estimated readily through initial reaction rate experiments. The constants α_1 and β_1 correspond to the intracellular key enzyme synthesis and degradation rates. As this key enzyme is hypothetical and not assayed, the estimation of these two constants is based on the synthesis and degradation rates of an average enzyme in the yeast cells. These two constants are also related to the maximum intracellular mass fraction of an average enzyme in yeast cells, which is estimated from the literature. The constants α_2 and β_2 relate to the synthesis and degradation rates of the extracellular β-glucosidase. As this enzyme is assayed, its maximum synthesis rate α_2 and its degradation rate β_2 are readily determined. The enzyme synthesis saturation constants K_{e_1} and K_{e_2} are more difficult to determine but can be estimated through curve fitting the initial lag during the growth on single substrates at several different initial concentrations. The constants K_3 and K_4 are the most difficult to determine and they are estimated through curve fitting the sugar concentration profile in the perturbed batch cultures(for K_3) and in

the simple batch cultures(for K_4) using various combinations of glucose and cellobiose concentrations.

The kinetic model explicitly includes terms for the repression/induction and activation/inhibition of key enzymes for substrate catabolism. Even this simple representation of complex internal regulatory mechanisms introduces parameters which are difficult to measure.

The complexity of the biochemical pathways constituting the regulatory mechanisms in the microbial cell present obvious difficulties when utilizing the kinetic modeling approach with any degree of thoroughness. The inclusion of terms representing phenomena such as catabolite repression is necessary if the kinetic model is to exhibit even a qualitative accuracy. However, the quantity and quality of the kinetic parameters rapidly increase in number and obscurity when additional pathways are represented in a kinetic model.

While striving for quantitative accuracy, an important goal of useful modeling techniques, kinetic modeling of biological systems quickly strays from the oversimplified Monod and Michaelis-Menten expressions. The major difficulty arising from including all known pathways associated with a biochemical event is that numerous chemical species must be included along with appropriate kinetic parameters. The concentrations of these species and parameters associated with their reactions are difficult to measure. Modeling assumptions which limit the number of experimentally determined parameters while not sacrificing qualitative or quantitative accuracy are valuable tools to be used, if possible, when model simplicity is desirable. The cybernetic approach to modeling biological systems utilizes such a tool.

Cybernetic Modeling

The cybernetic modeling approach views the microbial cell as an optimal strategist allocating a limited resource in order to maintain some optimal condition. The form of the resource need not be specified explicitly since the instantaneous strategy of optimization requires that the fractional allocation of this hypothetical resource must equal the fractional return from that allocation [3]. Therefore, only the form of the return, i.e., the quantity the cell is assumed to be maximizing needs to be specified. The resource has been hypothesized to be amino acids required for translation of key catabolic enzymes [5] and total transcription time allocated for transcribing genes coding for these enzymes [3]. Explanations of what this hypothetical resource physically represents are interesting, but the simplicity inherent in the cybernetic approach and the matching law result preclude the practical value of such explanations since the cybernetic model does not represent the mechanisms of resource allocation. The actual specification made for what the return will be in the model must be chosen based on experimental observations, and is not an unalterable feature of the cybernetic framework. The specific growth rate is the simplest choice for the return since researchers have repeatedly observed that the substrate supporting the faster growth is utilized preferentially to substrates supporting slower growth rates [7]. In addition, specific growth rate is easy to estimate through optical density measurements and can be accurately represented by a typical Monod form as was shown for the kinetic model;

$$\frac{dC}{dt} = \mu e_1 v_1 C \left[\frac{s_1}{K_1 + s_1} \right] \tag{8}$$

where the cybernetic variable, v_1, represents inhibition/activation of the key enzyme for glucose catabolism, e_1, such that $0 \leq v_1 \leq 1$. For example, if $v_1 = 0$, e_1 is totally inhibited and if $v_1 = 1$, e_1 is at maximum activity. The rate equation for e_1 is written as:

$$\frac{de_1}{dt} = \alpha_1 u_1 - e_1 \left(\frac{1}{C} \frac{dC}{dt} \right) - e_1 \beta_1 \tag{9}$$

where u_1 controls the induction/repression of e_1 synthesis such that $0 \leq u_1 \leq 1$. More specifically, u_1 represents the fractional allocation of some limited resource needed for the synthesis of e_1 and is defined in terms of a fractional return. The Monod type induction term containing K_{e_1} in equation 2 has been replaced by u_1 in the cybernetic model. The rate equation for glucose is analogous to the kinetic version with the cybernetic variables v_1 and v_2 incorporated for implicit activation/inhibition of e_1 and e_2 respectively.

$$\frac{ds_1}{dt} = -\frac{\mu e_1 v_1 C}{Y} \left[\frac{s_1}{K_1 + s_1} \right] + \nu k_2 e_2 v_2 \left[\frac{s_2}{K_2 + s_2} \right], \tag{10}$$

The non-competitive inhibition term of equation 5 containing K_3 has been replaced by v_2. As expected, the induction and repression terms containing the constants K_{e_2} and K_4 are replaced by the cybernetic variable, u_2, for the rate equation of e_2.

$$\frac{de_2}{dt} = \alpha_2 u_2 C - e_2 \beta_2 \tag{11}$$

Previously, the return has been specified as a rate of growth [3]. In the present system, the return is specified as the rate of substrate utilization. The rate of substrate utilization and growth rate are simply related by the cellular yield. Therefore, the rate of growth and rate of substrate utilization are essentially the same when choosing the quantity the cell is assumed to be maximizing. However, if growth rate is chosen, each substrate must have a corresponding maximum specific growth rate. For the glucose-cellobiose system where cellobiose is hydrolyzed extracellularly, the specific growth rate depends only on the glucose concentration. Hence, the rate of substrate utilization was chosen as the form of the return in order to differentiate glucose growth from cellobiose growth. The rate of glucose utilization is defined as:

$$r_1 = \frac{\mu e_1 v_1 C}{Y} \left[\frac{s_1}{K_1 + s_1} \right] \tag{12}$$

and the rate of cellobiose utilization is defined as:

$$r_2 = k_2 e_2 v_2 \left[\frac{s_2}{K_2 + s_2} \right] \tag{13}$$

Thus, the fractional returns can be written as:

$$u_1 = \frac{r_1}{r_1 + r_2} \tag{14}$$

$$u_2 = \frac{r_2}{r_1 + r_2} \tag{15}$$

The cybernetic variables, v_i, which control enzyme activation/inhibition are written as: [8]

$$v_1 = \frac{r_1}{max_i(r_1, r_2)} \tag{16}$$

$$v_2 = \frac{r_2}{max_i(r_1, r_2)} \tag{17}$$

Simulation Results

Figure 3 shows simulation results from both the kinetic and cybernetic models. Several characteristics of the experimental data are represented. The first is the sequential utilization of first glucose then cellobiose. This results from the explicit glucose inhibition of β-glucosidase in the kinetic model and the implicit inhibition in the cybernetic model. The second feature is the change in the rate of exponential growth at the point of glucose depletion. Since the growth rate depends on only the glucose concentration in both models, eqns 1 and 8, the lower glucose concentration during cellobiose hydrolysis accounts for the lower growth rate. Thirdly, the glucose concentration increases after its initial depletion. Although the modeling assumption of extracellular cellobiose hydrolysis assumes all hydrolysis occurs outside of the cell, the magnitude of the increase for the simulations is lower than from the experimental data. The reason for this discrepancy in the magnitude of glucose accumulation is not clear. Incomplete parameter evaluation could be the major reason. Although it is possible that due to model simplifications, neither the kinetic nor the cybernetic model can accurately represent this aspect of the system in their present form.

Figure 4 shows simulation results from both models for perturbed batch simulations where glucose is suddenly added during exponential growth in the presence of only cellobiose. The experimental features represented are an immediate increase in the rate of exponential growth upon glucose addition, and a decrease in the rate of cellobiose hydrolysis due to inhibition of β-glucosidase by the increased glucose concentration.

The final set of simulations in figure 5 is for growth on cellobiose only. Both the cybernetic and kinetic simulations show the increase in the glucose concentration presumably due to extracellular cellobiose hydrolysis. These simulations agree fairly well with the experimental data shown in figure 2.

Conclusions

Both the kinetic and cybernetic models contain gross oversimplifications of microbial regulatory mechanisms. Such simplifications are necessary for practical modeling purposes due to the immense complexity of the actual biochemical pathways constituting the regulatory mechanisms.

The distinguishing feature between the kinetic and cybernetic models is that the cybernetic model assigns an invariant strategy to the cell which is assumed to be sought by the cell's regulatory mechanisms at all times. This teleological approach is the basis of all modeling techniques categorized as cybernetic modeling and results in fewer parameters which must be determined experimentally.

In view of the oversimplifications made in both models presented here, there is a danger of creating parameters which become vague in their physical meaning or may not correspond to any phenomenon occurring within the microbial cell. Even in view of such cautions, simulations from both models have indicated a good agreement with the experimental data. Although this agreement is still at a qualitative level, there is little reason to suspect that further parameter evaluations can not render both models qualitatively and quantitatively more accurate.

References

[1] T.K. Ghose and K. Das, "A simplified kinetic approach to cellulose-cellulase system," *Adv. Biochem. Engr.*, **1**, 55-76(1971).

[2] S.M. Lastick, D. Spindler, S. Terrell, and K. Grohmann, "Simultaneous Saccharification and Fermentation of Cellulose," presented at *Biotech '84 USA*, Online Publications, Pinner, UK, pp. 277-289.

[3] Dhinakar S. Kompala, Doraiswami Ramkrishna, and George T. Tsao, "Cybernetic Modeling of Microbial Growth on Multiple Substrates," *Biotechnol. Bioeng.*, **26**, 1272-1281(1984).

[4] D. Ramkrishna, "A cybernetic perspective of microbial growth," in *Foundations of Biochemical Engineering: Kinetics and Thermodynamics in Biological Systems*, E. Papoutsakis, G.N. Stephanopoulos, and H.W. Blanch, Eds. (American Chemical Society, Washington, DC, 1982).

[5] P. Dhurjati, D. Ramkrishna, M.C. Flickinger, and G.T. Tsao, "A Cybernetic View of Microbial Growth: Modeling of Cells as Optimal Strategists," *Biotechnol. Bioeng.*, **27**, 1-9(1985).

[6] Difco Manual, "Dehydrated Culture Media and Reagents for Microbiology," (1984).

[7] D. Ramkrishna, D.S, Kompala, and G.T. Tsao, "Are Microbes Optimal Strategists?" *Biotechnology Progress*,(in print)

[8] D.S. Kompala, "Bacterial growth on multiple substrate: Experimental verifications of Cybernetic Models," Ph.D. Dissertation, Purdue University, 1984.

[9] A.G. Fredrickson, "Formulation of Structured Growth Models, *Biotechnol. Bioeng.*, **18**, 1481-1486(1976).

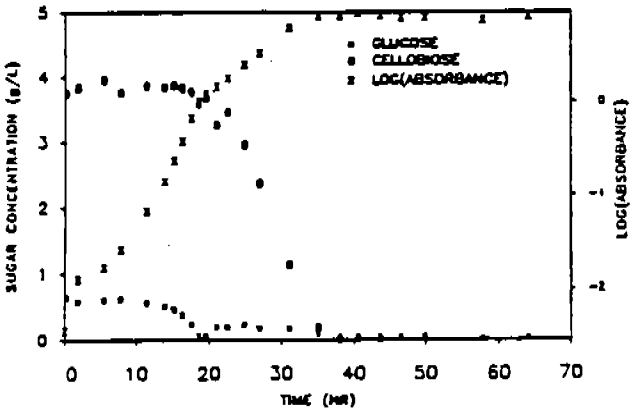

Figure 1: Batch Growth on Glucose and Cellobiose

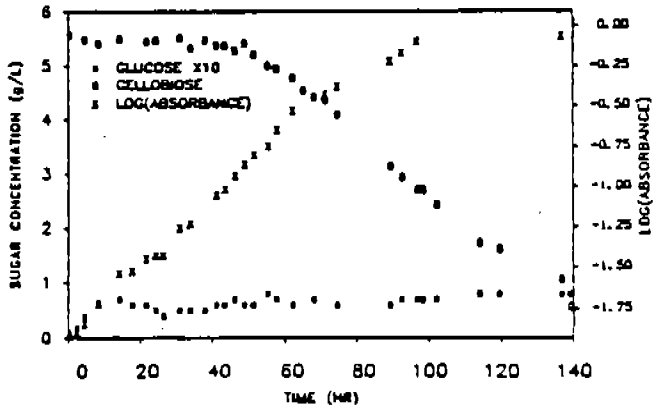

Figure 2: Batch Experiment with only Cellobiose added

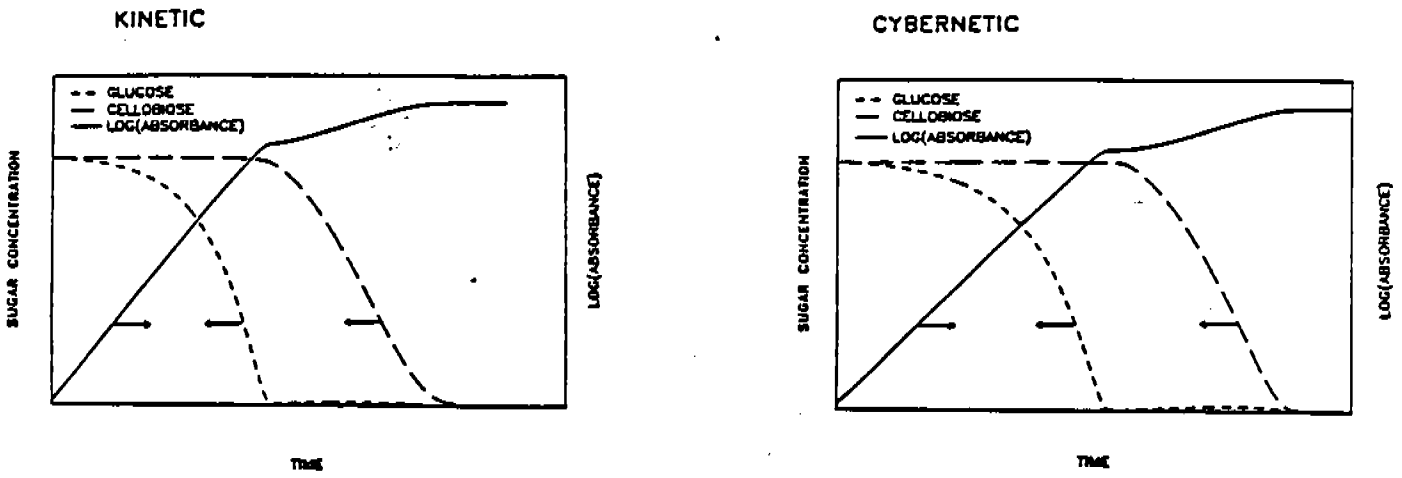

Figure 3: Kinetic and Cybernetic Simulations of Batch Growth Experiment

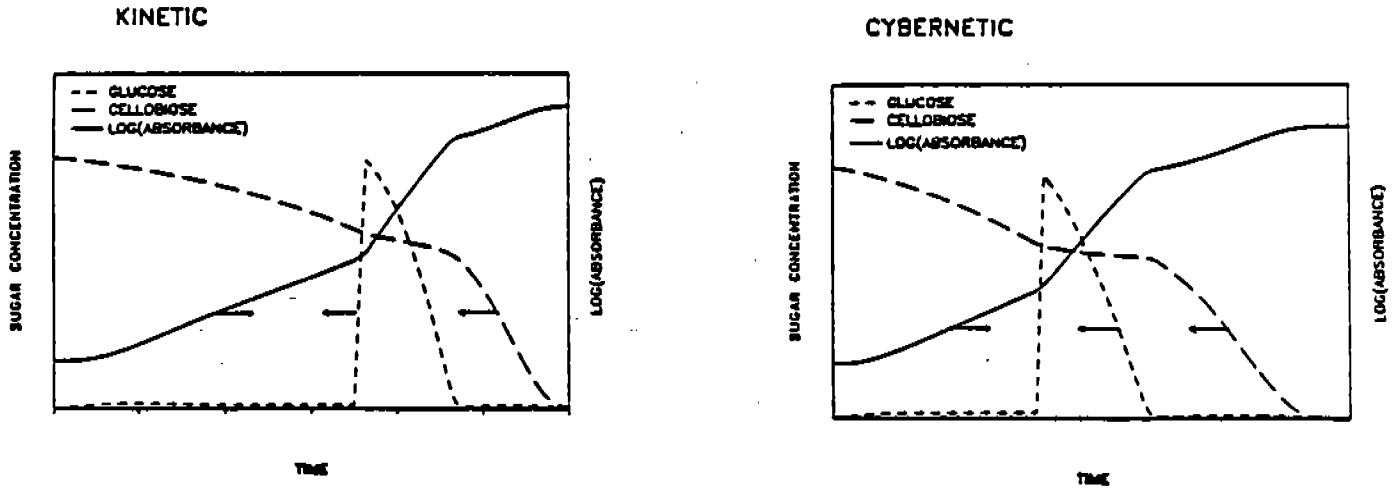

Figure 4: Kinetic and Cybernetic Simulations for Perturbed Batch Experiment

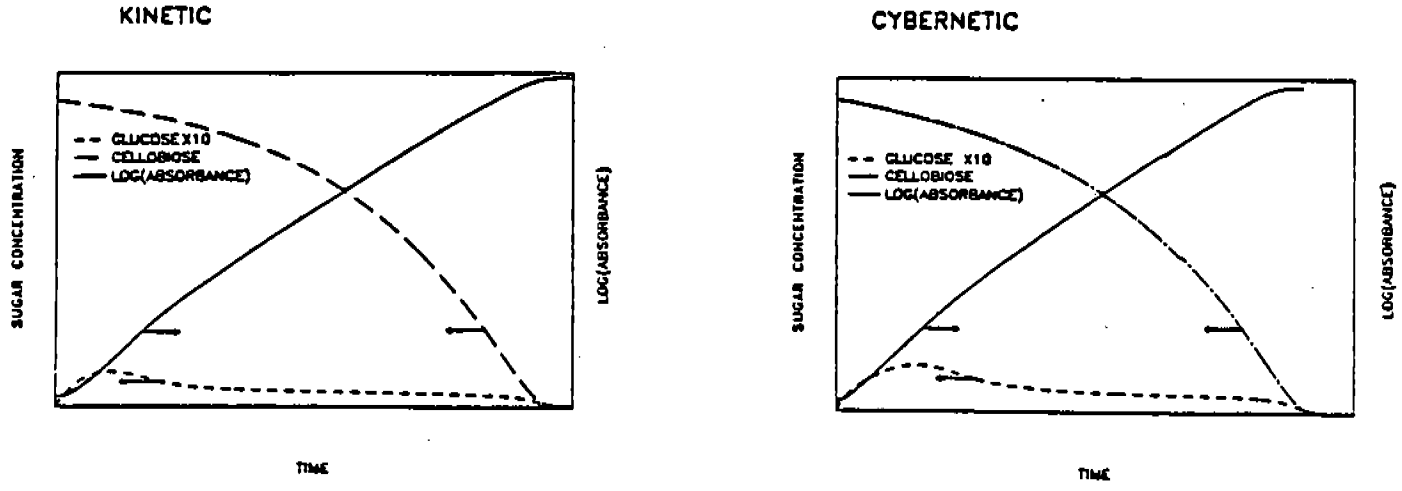

Figure 5: Kinetic and Cybernetic Simulations with only Cellobiose Added